IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Tanzi and Kim

Appl. No. 09/065,902

Filed: April 24, 1998

For: **Purified 20 kDa Presenilin 2 C-Terminal Fragment and Methods of Screening for Compounds That Inhibit Proteolysis of Presenilin 2**

Art Unit: 1644

Examiner: Clemens, K.

Atty Docket: 0609.4270001/REF/JUK

Declaration of Co-Inventors Under 37 C.F.R. § 1.132

Commissioner for Patents
Washington, DC 20231

Sir:

We, Rudolph E. Tanzi and Tae-Wan Kim, do declare and state that:

1. We are the co-inventors of the claimed invention in the captioned application.

2. In a rejection under 35 U.S.C. § 103(a), the Examiner cited Tanzi *et al.*, a publication entitled "The Gene Defects Responsible for Familial Alzheimer's Disease," published 1996 in *Neurobiology of Disease* 3:159-168. Tanzi *et al.*, at page 164, left column, discusses work described in Kim *et al.*, an abstract entitled "Proteolytic Processing of Wild-type and Mutant Forms of Presenilin 2," which published July 1996 in *Neurobiology of Aging* 17:S155, for the Fifty International Conference on Alzheimer's Disease and Related Disorders held July 24-29, 1996 in Osaka, Japan. A copy of Kim *et al.* is attached.

3. We, together with other workers (Olivia G. Hallmark, Warren H. Pettingell, and Wilma Wasco), co-authored Kim *et al.* The other persons listed on Kim *et al.* are co authors, but are not co-inventors of the subject matter of the captioned application. Olivia

Hallmark was a research assistant who provided technical assistance for the cell culture experiments. Warren Pettingell was a research technologist who provided technical assistance for the recombinant DNA experiments. Wilma Wasco was an assistant professor who provided the cDNA for presenilin 2, a reagent used for experiments as indicated in the captioned application. Therefore, these three co-authors of Kim *et al.* were merely working under our direction, providing technical assistance, or providing a reagent used in the experiments. These three co-authors did not make any inventive contribution to the claimed invention of the captioned application.

4. We further declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

7/26/00
Date

Rudolph E. Tanzi, Ph.D.

7/26/00
Date

Tae Wan Kim, Ph.D.

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Attachment: Kim *et al.*, *Neurobiology of Aging 17*:S155 (July 1996)

inhibition, and these toxic effects were compromised by the microtubule stabilizer paclitaxel (taxol). In cells treated with okadaic acid, a decrease of glutaminated tubulin and an increase of tyrosinated tubulin, i.e. a shift towards labile microtubules was seen. Furthermore MAP1B and MAP2 were degraded. Okadaic acid increased the levels of phosphorylation of tau at Ser396/404 sites, but not at Ser199/202 sites. While the PP-2A and PP-1 activities decreased, mitogen activated protein kinase (MAPK), cdc2 kinase and cyclin dependent kinase (CDK) 5 activities, but not glycogen synthase kinase 3 activity, increased. Neither an effect of PP-2B inhibitors in toxicity and tau phosphorylation, nor the expression of PP-2B were observed in the SY5Y cells. Taken together, these results suggest (1) that okadaic acid-induced cytotoxicity is partly related to the unstable microtubule system, including degradation of MAP1B and MAP2, and (2) that okadaic acid induced hyperphosphorylation of tau is the result of activated MAPK and CDKs, and the decreased activities of PP-2A and PP-1. (Supported by NIH grants AG05892, AG08076, NS18105 and Zenith Award from Alzheimer's Assoc., USA).

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Apoptosis in neurodegeneration. Walking the small line between life and death.

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There is increasing evidence that a population of neurons may die by the mechanism of apoptosis or programmed cell death in different neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease and Amyotrophic lateral Sclerosis. An interesting aspect of apoptosis is the induction of early-response genes, such as c-fos, c-jun and cyclin D, suggesting an frustrated attempt for postmitotic neurons to start mitosis. Moreover in AD, a number of candidate kinases intervening in aberrant tau phosphorylation are known to interfere with mitogenic pathways. In addition, an important issue is the relation between microtubule instability, induced by aberrant tau phosphorylation and the abortive mitotic cycle.

We therefore investigated the relationship between the kinase/phosphatase activity balance and neuronal apoptotic cell death in both proliferative and fully differentiated conditions.

In a model of nerve-growth factor deprivation, we document the appearance of aberrant phosphorylation of tau in relation to other markers of apoptosis (DNA fragmentation, membrane modifications) by quantitative immunocytochemistry and flow cytometry. Inhibition of kinase activity by the general protein kinase inhibitor staurosporine was shown to both decrease apoptotic cell death and aberrant tau phosphorylation and to modulate other apoptotic markers.

We compared this type of neurotoxicity with a model based on okadaic acid induced cell death in the proliferative human TR14 and in the fully differentiated, postmitotic NT2-N neuroblastoma cells. Time-lapse videomicroscopy and quantitative fluorescence microscopy using the membrane permeable nucleic acid marker SYTO was used to document different aspects of the apoptotic process. It was shown that okadaic acid forced both types of cells into an aberrant mitotic form. The cell cycle did not progress beyond prophase and bifurcated rapidly into an apoptotic process. This was followed inevitably by cellular swelling and membrane breakdown. This process was studied in relation to aberrant phosphorylation of tau protein and intracellular localization of cyclin dependent kinase 5(cdk5) and its activator protein p35.

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Apolipoprotein E-Derived 22 kDa Fragment Neurotoxicity: A New Pathophysiological Mechanism in Alzheimer's Disease

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The unexpected association of apoE phenotype with the risk of Alzheimer's Disease has given rise to a number of hypotheses implicating apoE in the pathology of this disorder. Most of these hypotheses are based on the assumption that apoE contributes in some way to the neurotoxicity of ß-amyloid or that isoforms of apoE differentially affect the stability of the neuronal cytoskeleton to alter their response to injury. The demonstration that peptide sequences including the receptor-binding domain of apoE, although not apoE itself, exhibit significant neuronal toxicity in vitro (Exp. Neurol. 130:120-126, 1994) suggested the alternative hypothesis that apoE plays a direct role in AD neuropathology as a source of neurotoxic peptides. One line of evidence supporting this hypothesis comes from the demonstration that proteolytic fragments of apoE are present in human brain and CSF samples (Marques et al., 1995), the most abundant of which is a 22 kDa fragment thought to be analogous to the major *N*-terminal thrombin cleavage product.

In the present study, the toxicity of the 22 kDa fragments derived from the E3 and E4 isoforms of apoE were tested. ApoE produced by transfected HEK cells (expressing either human apoE3 or apoE4) was concentrated from the culture medium, purified on a heparin column and then subjected to enzymatic cleavage with 1% thrombin for 24 hours. The 22 kDa fragments were subsequently purified using gel filtration then tested for toxicity. Embryonic chick sympathetic neurons were plated overnight in 96-well plates and then exposed overnight to various concentrations of the 22 kDa fragments derived from the E3 and E4 isoforms or to vehicle solutions. Significant neuronal toxicity was obtained with the 22 kDa fragments but not with vehicle solutions. Most importantly, the 22 kDa fragment derived from the E4 isoform was significantly more toxic than the same fragment derived from the E3 isoform. These results demonstrate that the major proteolytic fragment of apoE found in the brain is neurotoxic and that there are isoform-specific differences in such toxicity. The demonstration of greater toxicity of the 22 kDa fragment obtained from the E4 isoform supports the possibility that the genetic association of the E4 allele with the risk of AD may be directly related to the neuronal toxicity associated with this 22 kDa fragment. (Supported by NIH grant NS31410, UC Research Challenge Award and a MERF grant from the Mayfield Clinic.)

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Proteolytic Processing of Wild-type and Mutant Forms of Presenilin 2

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The majority of early-onset familial Alzheimer's disease (FAD) appears to be caused by mutations in two recently identified genes: presenilin 1 (PS1) and presenilin 2 (PS2). These two novel genes, PS1, located on chromosome 14, and PS2, on chromosome 1, are significantly homologous to each other and are members of an evolutionarily conserved gene family. The predicted structures of PS1 and PS2 contain six to nine hydrophobic domains, which produces several large and small hydrophilic loops. Neither the first eighty amino acids nor the single large hydrophilic loop are particularly well conserved, suggesting that these regions impart specificity of function or localization to the PS1 and PS2. The normal biological role(s) of the presenilins and the mechanism(s) by which the FAD-linked mutations exert their effect remains unknown. In the present study, we focused on PS2. To begin investigating normal cellular functions of PS2, we examined biosynthesis and processing of this molecule. For regulated expression of PS2 in human neuronal cells, we have established inducible cell lines expressing either wild-type or mutant forms of PS2 under the tight control of the tetracycline-responsive transactivator. In this system, presence of tetracycline in the culture medium supresses PS2 expression, while its withdrawal results in induction of PS2 expression. Western blot analysis revealed that either N-terminal or C-terminal FLAG epitope-tagged PS2 molecules were visualized as single bands with apparent molecular weights of 52 kDa. In addition to the 52 kDa species, we also observed high molecular weight aggregates and a 20 kDa C-terminal fragment. We examined whether the 20 kDa polypeptide is a stable cellular fragment or a non-specific degradation product. Both pulse-chase experiments and cyclohexamide treatment showed that the 20 kDa fragment was a stable cellular polypeptide and predominantly localized to the detergent insoluble fraction. In contrast, the 52 kDa PS2 product was completely extracted by detergent treatment. Our studies demonstrated that PS2 undergoes proteolytic processing to generate a 20 kDa derivative which is associated with the detergent insoluble fraction in the cell (e.g. cytoskeleton). Interestingly, generation of the 20 kDa fragment from mutant forms of PS2 was increased several fold compared to wild-type. These results raise the possibility that the PS2-derived C-terminal fragment is a byproduct of the normal cellular breakdown of PS2. This fragment may serve as a functional cellular effector and represent a potential marker for PS2-linked FAD neuropathogenesis.

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The study on trace elements in brains of dementia patients

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Aluminum(Al) neurotoxity and its relevance to the cause of dementias have been an important issue in recent years. The neutron activation analysis, a non-destructive method, was applied to determine the concentrations of seven elements(Cu, Al, Co, Mn, P, Zn and Fe) in the brain specimens from dementia patients and their controls. A correction was performed for the $^{31}P(n,\alpha)^{28}Al$ fast neutron primary interference reaction in the Al determination. This study includes these two groups: Japanese cases(10 Alzheimer's disease(AD), 1 Pick's disease and 8 control cases); and Guamanian cases(3 Parkinsonism-dementia complex(PD), 1 amyotrophic lateral sclerosis and 4 non-demented control cases). The results obtained were as following: